THE PHOTOSYNTHETIC RHYTHM OF ACETABULARIA CRENULATA. II. MEASUREMENTS OF PHOTOASSIMILATION OF CARBON DIOXIDE AND THE ACTIVITIES OF ENZYMES OF THE REDUCTIVE PENTOSE CYCLE

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Research in biological rhythms has brought to light a large number of cases in which highly integrated physiological processes in both higher and lower plants are regulated periodically by an endogenous time-keeping mechanism. Recently a number of investigations have sought to reveal the point at which control is exercised by analyzing the time-dependence of key component steps. For example, Richter and Pirson (1957) found diurnal rhythms in the activities of acid phosphatase, catalase and phosphorylase in cell extracts of the green alga *Hydrodictyon* that correlated positively with diurnal changes in photosynthetic and respiratory rates. However, no such changes were discerned in the activities of aldolase and triosephosphate dehydrogenase, enzymes that are known to be more specifically involved in photosynthetic and respiratory pathways. Khan and Sanwal (1965) in studying diurnal fluctuations in the organic acid content of the cactus, *Nopalea dejecta,* discovered parallel periodicity in three enzymes of the tricarboxylic cycle.

Studies on the marine dinoflagellate, *Gonyaulax polyedra,* have been particularly interesting because of the apparent directness and simplicity of the mode of periodic control in this organism. Hastings *et al.* (1956 and 1962) showed that the rhythm in bioluminescence may be attributed in part to concurrent changes in the amount of luciferin and in the activity of luciferase in cell-free extracts. *Gonyaulax* also exhibits a marked circadian rhythm in photosynthetic capacity when cells are maintained in dim light and exposed periodically to a saturating intensity for brief intervals (Sweeney, 1960). The fact that no rhythmicity is expressed at rate-limiting light intensities suggested that dark reactions, only, are affected by the control mechanism. Subsequent investigation revealed that the Hill reaction of whole cells in the presence of quinone and the sensitivity to uncouplers of photophosphorylation do not undergo diurnal variation (Sweeney, 1965). On the other hand, the activity of ribulose diphosphate carboxylase in cell extracts varied in a diurnal cycle, the amplitude of which was sufficient to account for the observed rhythm in photosynthetic capacity. Sweeney's paper appears to be the first report of a close parallel in a photosynthetic rhythm and the activity of an enzyme known to be essential to the photoassimilation of CO_2, though the data unfortunately do

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not permit a quantitative comparison between the rate of photoassimilation and the activity of the enzyme.

In the present work we have undertaken such comparisons using the slowly developing giant alga, *Acetabularia crenulata,* which is known to possess a pronounced photosynthetic rhythm (Sweeney and Haxo, 1961; Richter, 1963). Cells taken from the light and dark phases of a daily cycle have been compared in their ability to photoassimilate CO_2 at low and high light intensities and in the activity of 9 enzymes in the reductive pentose phosphate pathway. *Acetabularia* cells expand logarithmically for a number of weeks without cell division before undergoing morphogenesis (Terborgh and Thimann, 1964) and so possess the inherent advantage that their photosynthetic rhythm is uncomplicated by concurrent biochemical changes associated with differentiation and a cell division cycle.

Materials and Methods

The experimental material consisted of *Acetabularia crenulata* Lamoroux cells in the phase of stalk elongation. The conditions of growth were the same as described in the preceding paper (Terborgh and McLeod, 1967). The cultures were given a regimen comprised of alternating 8-hour light and 16-hour dark periods for at least two weeks prior to experimentation. The light source was a battery of "Daylight" fluorescent tubes that gave an illuminance of 3 klux at the level of the culture flasks. Experimental cells were conditioned in two chambers in which the light-dark cycles were 180° out-of-phase. Samples were collected simultaneously from the two chambers, and will be referred to as L (middle of light phase) and D (middle of dark phase) samples. Portions of the samples were then immediately used for measurements of photoassimilatory rates, fresh weight and chlorophyll determinations, and for the preparation of homogenates for enzyme analyses.

Measurements of photoassimilation employed approximately 50-mg. fresh weight portions of *Acetabularia* cells. These were suspended in square 4-ml. cuvettes containing 3 ml. of medium that held a known amount of $NaHCO_3$. The cuvettes were covered with Parafilm and exposed to illumination in a water bath at 25° C. The light source was a tungsten bulb, from which most of the heat was eliminated by passing the beam through a tank of water. The light intensity at the position of the cuvettes was varied by interposing neutral density filters, and/or varying the distance of the light source. At the beginning of the experiments 2 μC. of $NaHC^{14}O_3$ were added to the cuvettes, and after 15 minutes of photoassimilation of labelled bicarbonate the cells were removed from the cuvettes, rinsed in fresh medium, blotted lightly, and transferred to vials containing 5 ml. of methanol. The cells were then ground in a glass homogenizer with a small amount of glass-fiber paper added to facilitate the grinding. After homogenization, 25 μl. of 1 N HCl were added, and air was bubbled through the suspension for about three minutes to eliminate $C^{14}O_2$ not assimilated by the cells. Duplicate 100-μl. portions of the acidified homogenates were plated and the radioactivity determined with a Nuclear-Chicago model D47 gas-flow counter of known efficiency. Self-absorption of β-emission was negligible for the small amounts of solid material plated on each planchet. Chlorophyll *a* determinations were performed on portions of the homogenate, after removal of suspended particles by centrifugation, according to the method of Bruinsma (1961).

Enzyme activity determinations used approximately 200-mg. fresh-weight portions of the L and D cultures. The cells were blotted on moist filter paper and transferred to a glass homogenizer to which were added 2 ml. of 0.01 *M* Tris, pH 7.8, and a small piece of glass-fiber filter paper to facilitate the grinding. Following homogenization for about two minutes at 0° a portion of the homogenate was used for chlorophyll *a* determination, and the rest centrifuged at 0° and 30,000 *g* for 10 minutes. Portions of the supernatant of the centrifuged homogenate were then subjected to enzyme activity analyses as described below.

Most of the assays involved directly, or were coupled to, the oxidation or reduction of the nicotinamide adenine dinucleotides. These assays were carried out in a total volume of 1 ml. in 1-cm. cuvettes. The change in absorbance at 340 mμ was recorded with a Beckman DK ratio-recording spectrophotometer against a distilled water blank. The amount of supernatant from the cell homogenates used as an enzyme source was adjusted to give rates of change or absorbance of 0.1 to 0.5 optical density units per minute. Controls from which substrate was omitted were always run. The methods used for the various enzyme assays have been described previously by Campbell, Hellebust and Watson (1966).

RuDP carboxylase activity was determined from the amount of radioactivity incorporated when the extract was incubated with $NaHC^{14}O_3$ and RuDP, by a modification of the methods of Horecker, Hurwitz and Weissbach (1956). The reaction mixture contained 25 μmoles of Tris (pH 7.8), 0.05 μmoles of $NaHCO_3$, 1 μC. of $NaHC^{14}O_3$, 1.5 μmoles of RuDP, and extract, in a final volume of 250 μliters. After 10 minutes, the reaction was stopped with 25 μliters of 1 N HCl. The mixture was aerated to drive off unreacted CO_2, a 25-μliter sample was plated and dried, and its radioactivity was counted with a Nuclear-Chicago model D47 gas-flow detector.

Five μmoles of R-5-P and 5μmoles of adenosine triphosphate were substituted for RuDP in the RuDP assay system for the measurement of overall activity of R-5-P isomerase, and RuDP carboxylase.

The problem of aliquoting *Acetabularia* into replicate samples for the various types of analyses was solved by using approximately the same number of cells per sample, and then using the chlorophyll content, obtained from determinations of aliquots of homogenates of the samples, as a common denominator.

Abbreviations. R-5-P = ribose-5-phosphate, Ru-5-P = ribulose-5-phosphate, RuDP = ribulose-1.5-diphosphate, FDP = fructose-1.6-diphosphate, P6A = 3-phosphoglyceric acid, 6-3-P = glyceraldehyde-3-phosphate, $NADH_2$ = reduced nicotinamide-adenine dinucleotide, $NADPH_2$ = reduced nicotinamide adenine dinucleotide phosphate.

Results

A first experiment established the relation between light intensity and rate of photoassimilation of CO_2 for cells taken from the middle of the light and dark phases. Figure 1 shows that the photoassimilatory rates of the L sample were considerably higher than those for the D sample at light-limiting as well as at light-saturating intensities. At 500 lux (light-limiting) the rate of the L cells is about 60%, and at 15,000 lux (light-saturating) about 72% higher than the rates for D cells for the corresponding light intensities. Similar data on the relationship

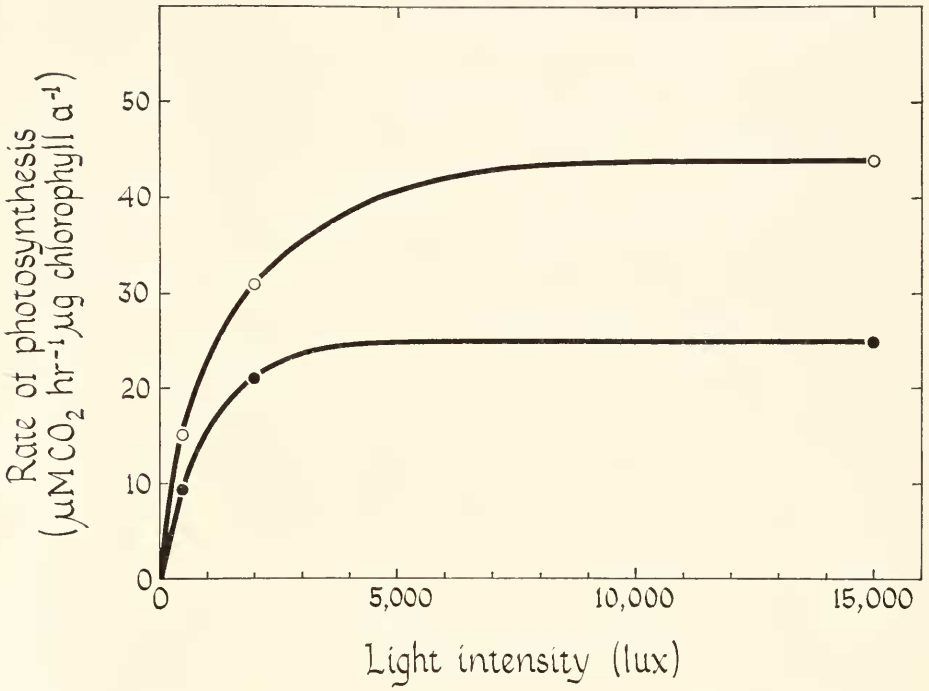

FIGURE 1. Light curve for photoassimilation of CO_2 by *Acetabularia* cells grown on a regime of 8 hours of light at 3000 lux and 16 hours of darkness. The curve with closed circles represents cells harvested at the middle of the dark period, and the curve with open circles represents cells from the middle of the light period.

TABLE I

*Rates of photoassimilation of CO_2 and RuDP carboxylase activities of Acetabularia cells harvested at the middle of the light (L) and the dark (D) period**

	Experiment 1			Experiment 2		
	L	D	L/D	L	D	L/D
Rate of photoassimilation of CO_2 at the following light intensities:						
a) 500 lux	—	—		0.009	0.007	1.3
b) 2500 lux	0.021	0.014	1.5	0.012	0.008	1.5
c) 15,000 lux	0.024	0.017	1.4	0.018	0.009	2.0
RuDP carboxylase activity at the following substrate concentrations:						
a) 2×10^{-2} *M* HCO_3^-	0.029	0.026	1.1	0.040	0.035	1.1
b) 2×10^{-3} *M* HCO_3^-	0.009	0.007	1.3	0.012	0.010	1.2

* All the figures represent μmoles CO_2 $hr.^{-1}$ [μg. chlorophyll *a*]$^{-1}$.

between light intensity and photoassimilation rates for L and D cells are also presented in Tables I, II, and III, and confirm on the whole the relationship presented graphically in Figure 1.

Two experiments correlating the activities of RuDP carboxylase with rates of photoassimilation of CO_2 are recorded in Table I. While the photosynthetic rates of the L cells were consistently higher at all three light intensities than the corresponding rates for D cells, the activities of RuDP carboxylase at two different substrate concentrations were only slightly, and probably not significantly, higher in the L cells than the D cells. Moreover, the observation that RuDP carboxylase from L and D cells responds similarly to changes in substrate concentration indicates that the enzymes from the light and dark cells are kinetically similar. At the higher bicarbonate concentration the activities of RuDP carboxylase are sufficient, or even in excess of those required to account for the corresponding rates of photoassimilation of CO_2 by the intact cells at light saturation (15,000 lux).

A similar experiment, that included assays of several additional enzymes involved in the reductive pentose phosphate cycle, is presented in Table II. Though the photoassimilation rates of L cells were 1.2 to 1.5 times greater than those shown by D cells, the activities of RuDP carboxylase in the extracts appeared to be identical. Similarly, the overall activities of R-5-P isomerase, Ru-5-P kinase, and RuDP carboxylase were also approximately the same for L and D cells. The low activity of this sequence relative to RuDP carboxylase alone was probably due to rate-limiting concentration of the intermediate substrates Ru-5-P and RuDP

TABLE II

*Rates of photoassimilation of CO_2 and activities of enzymes involved in the reductive pentose phosphate cycle of Acetabularia cells harvested at the middle of the light (L) and the dark (D) period**

	L	D	L/D
Rate of photoassimilation of CO_2 at the following light intensities:			
a) 500 lux	0.013	0.009	1.4
b) 2500 lux	0.017	0.014	1.2
c) 15,000 lux	0.022	0.015	1.5
Enzyme activities:			
RuDP carboxylase at			
a) 2×10^{-2} *M* HCO_3^-	0.037	0.037	1.0
b) 2×10^{-3} *M* HCO_3^-	0.010	0.011	0.9
Overall activity of R-5-P isomerase, Ru-5-P kinase, and RuDP carboxylase**	0.020	0.019	1.1
Phosphoglycerate kinase	0.88	0.80	1.1
Glyceraldehyde-3-P dehydrogenase:			
$NADH_2$-dependent	0.14	0.13	1.1
$NADPH_2$-dependent	0.06	0.06	1.0

*All the figures represent μmoles substrate $hr.^{-1}$ [μg. chlorophyll a]$^{-1}$. The substrates used for these calculations were as follows for the different reactions: photoassimilation and RuDP carboxylase, CO_2; aldolase, FDP; phosphoglycerate kinase, PGA; glyceraldehyde-3-P dehydrogenase, $NADH_2$ or $NADPH_2$.

** In the presence of 2×10^{-1} *M* HCO_3^-.

TABLE III

*Rates of photoassimilation of CO_2 and activities of enzymes involved in the reductive pentose phosphate cycle of Acetabularia cells harvested at the middle of the light (L) and the dark (D) period**

	L	D	L/D
Rate of photoassimilation of CO_2 at the following light intensities:			
a) 500 lux	0.006	0.006	1.0
b) 2500 lux	0.014	0.012	1.2
c) 15,000 lux	0.026	0.018	1.4
Enzyme activities:			
RuDP carboxylase*	0.032	0.030	1.1
Phosphoglycerate kinase	0.88	0.92	1.0
Glyceraldehyde-3-P dehydrogenase:			
$NADH_2$-dependent	0.11	0.13	0.8
$NADPH_2$-dependent	0.16	0.16	1.0
Triose isomerase	0.22	0.21	1.0
Aldolase	0.044	0.050	0.9
Transketolase	0.024	0.025	1.0
Transaldolase	0.033	0.030	1.1
R-5-P isomerase	0.22	0.24	0.9
Ru-5-P kinase	0.13	0.14	0.9

* All the figures represent μmoles substrate $hr.^{-1}$ [μg. chlorophyll a]$^{-1}$. The substrates used for these calculations were as follows for the different reactions: photoassimilation and RuDP carboxylase, CO_2; phosphoglycerate kinase, PGA; glyceraldehyde-3-P dehydrogenase, $NADH_2$ or $NAPH_2$; aldolase, FDP; transketolase and transaldolase, $NADH_4$; R-5-P isomerase, R-5-P; Ru-5-P kinase, Ru-5-P, and triose isomerase, G-3-P.

** In the presence of 2×10^{-2} *M* HCO_3^-.

during the relatively short (10-minute) reaction period, rather than to appreciably lower activities of R-5-P isomerase and Ru-5-P kinase relative to that of RuDP carboxylase. The activities of phosphoglycerate kinase, and glyceraldehyde-3-P dehydrogenase, both $NADPH_2$- and $NADH_2$-dependent, were also not significantly different for light and dark cells. The results indicate that the activities of all the enzymes tested were considerably higher than those required to account for the observed rates of photoassimilation of CO_2 at light saturation.

Since theoretically any enzyme required for the operation of the reductive pentose phosphate cycle could be the limiting factor for CO_2 assimilation at light saturation, we performed a final series of experiments that included assays for a total of 9 enzymes in the cycle (Table III). The data show clearly that the activities of none of these enzymes differ to a significant degree in extracts from L and D cells.

The activities of RuDP carboxylase, aldolase, transaldolase, and transketolase were of the same order of magnitude as the rate of photoassimilation of CO_2 at light saturation, while those of phosphoglycerate kinase, $NADH_2$ and $NADPH_2$-dependent glyceraldehyde-3-P dehydrogenase, triose isomerase, R-5-P isomerase, and Ru-5-P kinase were considerably higher than necessary to account for the observed rates of photoassimilation in either L or D cells. The activities of $NADPH_2$- and $NADH_2$-dependent glyceraldehyde-3-P dehydrogenase were of a

similar magnitude in this experiment, while the $NADH_2$-dependent activity was approximately twice as great as the $NADPH_2$-dependent activity in the experiment recorded in Table II.

Discussion

The data presented in this paper on photoassimilation of CO_2 by *Acetabularia crenulata* cells demonstrate that the photosynthetic rhythm is expressed at light-limiting as well as light-saturating intensities, although the amplitude of the rhythm is less pronounced at the lower light intensities. These results are in full agreement with those obtained by continuous polarographic oxygen measurements (Terborgh and McLeod, 1967), and strongly suggest that the factor(s) immediately controlling the rate of photosynthesis act at the level of light as well as dark reactions. A recent report by Hoffman and Miller (1966), demonstrating an endogenous rhythm in the Hill-reaction activity of isolated tomato chloroplasts, also suggests that the control of photosynthetic rhythms may involve light reactions. The regulation of a photosynthetic rhythm *via* any of the enzymes of the reductive pentose phosphate cycle would result in changes expressed only at the level of the dark reactions. A second controlling factor would thus have to be assumed to account for the observed rhythm at the light reaction level (quantum yield).

Extracts of *Acetabularia* cells taken from the middle of the light and the dark periods, show no significant difference in the activity and affinity of RuDP carboxylase. This enzyme thus does not appear to be the agent through which the photosynthetic rhythm is expressed as seems to be the case in *Gonyaulax polyedra* (Sweeney, 1965). Dark and light samples, moreover, did not differ significantly in the activities of any of eight other enzymes known to be required for the operation of the reductive pentose phosphate cycle. Five of these enzymes, phosphoglycerate kinase, glyceraldehyde-3-P dehydrogenase, triose isomerase, R-5-P isomerase, and Ru-5-P kinase, had activities considerably above those required to account for the observed rate of photoassimilation of CO_2 at light saturation, and for this reason are not likely to be involved in the control of the photosynthetic rhythm. The activities of RuDP carboxylase, aldolase, transketolase and transaldolase were of the order of magnitude required to account for the observed rates of photoassimilation; however, as already stated, no rhythm was expressed in the activities of these enzymes. All the measurements of enzyme activities were performed on all extracts and employed "optimal" assay conditions; *i.e.*, optimal with respect to pH, substrate concentration, redox conditions, metal ion concentrations, etc. Our results can only be taken to indicate that there is no rhythmic variation in the amount of the enzymes investigated, and in the case of RuDP carboxylase in its affinity for CO_2 (*in vitro*). On the other hand, it is entirely possible that the *activity* of any one of these enzymes could vary in a rhythmic fashion in the cell in response to rhythmic changes in the cellular environment, such as pH, redox potential, ion concentrations or substrate concentration, and therefore, in fact, be involved in the expression of the observed rhythm of photoassimilation of CO_2. Investigation of the possibility of such an *in vivo* rhythm of enzyme activity would necessarily involve measurements of relative turnover rates of pools of intermediate substrates. Unfortunately, experiments of this kind with marine algae present great technical difficulties because of their high salt contents.

Driessche (1966) has recently reported a circadian rhythm in chloroplast shape in *Acetabularia mediterranea*. In the middle of the light period, coincident with maximum rates of O_2 evolution, the chloroplasts become elongate. During the dark period, when O_2 evolution was at a minimum, the shape of the chloroplasts was more spherical. Thus, we now have three rhythms concerning photosynthesis or the chloroplasts of *Acetabularia* to account for: in photosynthetic dark reactions, photosynthetic light reactions (quantum yield), and chloroplast shape. Possibly the endogenous time-keeping mechanism of *Acetabularia* regulates only a single key photosynthetic reaction which in turn underlies two or more manifest oscillations. Such a conservative mechanism could account for parallel changes in photosynthetic activity and maximum capacity if the critical reaction were to moderate the activity or amounts of primary reductant or of the long-wave-length form of chlorophyll *a*. A certain amount of circumstantial evidence stands in accord with possibilities of this kind. Kok (1956) and later Kok, Glassner and Rurainski (1965) have shown with *Chlorella* and spinach chloroplasts that during reversible inhibition of photosynthesis by high light intensities the quantum yield was affected to exactly the same extent as the photosynthetic dark reactions. Kok (1956) has postulated a reversible inactivation of photosynthetic units that also affects the rate of dark reactions. Under quite a contrary set of conditions we found a concurrent deterioration of light and dark reactions in the green flagellate, *Dunaliella tertiolecta*, in response to prolonged darkness or to very dim light (Hellebust and Terborgh, 1967).

Since a relationship has been established between photosynthetic phosphorylation and the structural state of fragments of spinach chloroplasts (Packer, 1962), as well as light-dependent volume changes in chloroplasts *in vitro* (Packer *et al.*, 1965), it may be that the rhythms in photosynthesis and chloroplast shape have a common basis.

In conclusion our evidence indicates that the circadian oscillation in the activity and maximum capacity of photosynthesis in *Acetabularia* thus stands in contradistinction with *Gonyaulax* in which a rhythm in RuDP carboxylase activity may be sufficient to explain the observed rhythm in photosynthetic capacity. The apparent disparity in the control mechanisms of these two organisms can be rationalized from an evolutionary point of view if one makes the assumption that circadian rhythms, where found, have selective value. For plants of diverse ecological proclivities, having photosynthetic rhythms optimal adaptation might entail a fluctuation of maximum capacity as in *Gonyaulax*, in both activity and capacity as in *Acetabularia*, or in activity alone, a case for which we have no clear example at present.

Summary

1. The photosynthetic rhythm of *Acetabularia crenulata* affects both light (quantum yield) and dark reactions in a parallel manner.

2. No significant difference was found between the activity of RuDP carboxylase in the extracts of samples taken at the middle of the light and dark periods nor was any difference detected in the affinity of this enzyme to CO_2. The activity of RuDP carboxylase in the cell extracts was sufficient to account for the observed rates of photoassimilation of CO_2 at saturating light intensities.

3. The activities of eight other enzymes of the reductive pentose phosphate cycle were also shown not to differ to a significant extent in extracts of cells taken at the middle of the light and dark periods. Five of these enzymes (phosphoglycerate kinase, glyceraldehyde-3-P dehydrogenase, triose isomerase, R-5-P isomerase, and Ru-5-P kinase) had activities considerably above those required for the observed light-saturated rate of CO_2 assimilation while the activities of aldolase, transketolase and transaldolase were of the same order of magnitude as the observed rates of photoassimilation.

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